Signal transduction pathways of IL-1β-mediated iNOS in pulmonary vascular smooth muscle cells

JONATHAN D. FINDER,1 JENNIFER L. PETRUS,1 ANDREW HAMILTON,2 RAPHAEL T. VILLAVICENCIO,3 BRUCE R. PITT,4 AND SAID M. SEBTI5

Departments of 1Pediatrics, 3Surgery, and 4Environmental and Occupational Health, University of Pittsburgh School of Medicine and Graduate School of Public Health, Pittsburgh, Pennsylvania 15261; 5Drug Discovery Program, H. Lee Moffitt Cancer Center, Department of Biochemistry and Molecular Biology, University of South Florida, Tampa, Florida 33612; and 2Department of Chemistry, Yale University, New Haven, Connecticut 06520

Received 10 January 2001; accepted in final form 21 May 2001

Finder, Jonathan D., Jennifer L. Petrus, Andrew Hamilton, Raphael T. Villavicencio, Bruce R. Pitt, and Said M. Sebti. Signal transduction pathways of IL-1β-mediated iNOS in pulmonary vascular smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 281: L816–L823, 2001.—Interleukin (IL)-1β is an important early mediator of inflammation in pulmonary artery smooth muscle cells. We previously reported that a geranylgeranyltransferase inhibitor elevated basal levels of inducible nitric oxide synthase (iNOS) and enhanced IL-1β-mediated induction, suggesting that Rac or Rho small G proteins are candidates for antagonism of such induction. In this study, overexpression of constitutively active Rac1 or its dominant negative mutant did not affect IL-1β induction of iNOS. Alternatively, treatment with Clostridium botulinum C3 exoenzyme, which ADP-ribosylates Rho, was associated with superinduction of iNOS, suggesting an inhibitory role for Rho. IL-1β activated the three mitogen-activated protein kinase (extracellular signal-regulated kinases 1 and 2, c-Jun NH2-terminal kinase/stress-activated protein kinase, and p38) and the Janus kinase (JAK)-signal transducer and activator of transcription pathways. The former two pathways were not associated with IL-1β-mediated iNOS induction, whereas the latter two appeared to have inhibitory roles in iNOS expression. These data suggest that a broad intracellular signaling response to IL-1β in rat pulmonary artery smooth muscle cells results in elevated levels of iNOS that is opposed by the geranylgeranylated small G protein Rho as well as the p38 and JAK2 pathways.

inducible nitric oxide synthase; interleukin-1β; Rho; mitogen-activated protein kinase

INTERLEUKIN (IL)-1β is an important early mediator of inflammation in a variety of cell types including pulmonary artery smooth muscle. IL-1β mediates inflammation, at least in part, by inducing expression of inducible nitric oxide (NO) synthase (iNOS), with concordant enhanced synthesis of NO, a critical effector molecule. In particular, we have shown that IL-1β alone, in sufficient quantities, in cultured rat pulmonary artery smooth muscle cells (RPASMCs) is capable of 1) inducing iNOS (33, 47); 2) increasing production of superoxide anion as well as of NO, resulting in the production of peroxynitrite (7); and 3) producing sufficient NO to be toxic to cocultures of rat pulmonary artery endothelial cells (22, 43).

IL-1β results in activation of a complex array of intracellular signaling molecules (35) including several GTPases and serine/threonine kinases in a tissue- and cell-specific fashion (38, 39). Relatively little is known, however, regarding the mechanism by which IL-1β affects iNOS expression in pulmonary vascular smooth muscle. In this regard, we used novel synthetic isoprenoid inhibitors to show that small GTP-binding proteins of the Ras/Rho superfamily are involved in the regulation of iNOS (16) and the synthesis of superoxide anion (6) by IL-1β in RPASMCs. Inhibition of geranylgeranyltransferase resulted in superinduction of iNOS (16) and inhibition of superoxide anion (6), perhaps accounting for the inhibition in growth and promotion of apoptosis in these cells by IL-1β (40). In the present study, we focused on the role of Rho, a geranylgeranylated protein, that was coimmunoprecipitated with the IL-1 receptor after IL-1 stimulation in HeLa cells (39) and appeared to act as a negative regulator of iNOS expression in human tumor cell lines (19) and systemic vascular smooth muscle cells (32).

IL-1β stimulation has been associated with several of the mitogen-activated protein (MAP) kinase (MAPK) cascades involved in the transmission of signal from the cell surface to the nucleus. The main signaling cascades are referred to by their ultimate protein kinase (ERK) 1/ERK2 (p44MAPK/p42MAPK) kinase, p38 kinase, and c-Jun NH2-terminal kinase (JNK)-stress-activated protein kinase (SAPK). Although these pathways have not been demonstrated to be involved in IL-1β-mediated effects on pulmonary vascular smooth

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
muscle, it is noteworthy that p38 and ERK contribute to such effects in human (24) or canine (20) airway or myometrial (3) smooth muscle. Signal transducer and activator of transcription (STAT) proteins are critical signaling molecules in most cytokine-mediated responses. Activation of STAT proteins usually involves a cytokine receptor-associated kinase [Janus kinase (JAK)], with subsequent serine phosphorylation by less well-defined kinases (8). In general, it has not been possible to place STATs clearly in the MAPK cascade (12), although the coactivation and contributions of these pathways are evident. In particular, the JAKs, which act through the associated STAT proteins (9), have, until recently, not been associated with IL-1β signaling (10). Although the mechanism of STAT involvement in IL-1 signaling remains obscure, two recent reports (31, 44) have suggested an involvement of STAT in the IL-1 receptor signaling pathway. Both the MAPK and STAT pathways are components of the signaling pathways of IL-1β induction of iNOS, including p38 in cardiac myocytes (24), mesangial cells (18), chondrocytes (2) and islet cells (26); JNK/SAPK in mesangial cells (18); ERK1/ERK2 in rat islet cells (26) and cardiac myocytes (24); and JAK-STAT in interferon (IFN)-γ-primed rat islet cells (21). Accordingly, we examined the contributions of these complex pathways to iNOS regulation in RPASMCs.

MATERIALS AND METHODS

All experiments were performed in triplicate unless noted. Representative experimental results are shown.

Cell culture. RPASMCs were isolated from explants of the intrapulmonary arteries of adult male Sprague-Dawley rats. The explants were placed endothelium side down in flasks containing low-glucose DMEM Ham’s F-12 medium (1:1; GIBCO BRL, Life Technologies, Grand Island, NY), 10% fetal bovine serum (HyClone, Logan, UT), 4 mM L-glutamine, 5 U/ml of penicillin, and 5 µg/ml of streptomycin (Sigma, St. Louis, MO), grown to confluence, and subpassaged. The cells were confirmed as smooth muscle by positive immunostaining for the smooth muscle isoforms of actin and myosin.

Chemical inhibitors. The MAPK kinase 1 inhibitor PD-98059 was purchased from New England BioLabs (Beverly, MA) and was used at a concentration of 10 µM in all experiments. The JAK2 inhibitor AG-490 (tyrphostin) and the p38 inhibitor SB-203580 were purchased from Calbiochem (La Jolla, CA). AG-490 was used at a concentration of 10 µM for all experiments. SB-203580 was used at a concentration of 10 µM for all experiments. The geranyleranylation inhibitors GGTI-298 and GGTI-2166 and the farnsyltransferase inhibitor FTI-277 were produced by A. Hamilton. They were used at concentrations of 10, 15, and 10 µM, respectively.

Western blotting. The cell monolayers were lysed in ice-cold buffer (50 mM Tris, pH 8.0; 110 mM NaCl; 5 mM EDTA; 1% Triton X-100; and the protease inhibitors antipain, pepstatin, leupeptin, chymostatin, and phenylmethylsulfonyl fluoride (PMSF), scraped into 1.5 ml sample tubes, and centrifuged to remove cellular debris. Protein levels of the supernatants were then determined. After being boiled in Laemmli buffer, 8–25 µg of the whole cell extract were separated on 7.5% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with an affinity-purified rabbit polyclonal antibody to murine macrophage iNOS (Transduction Laboratories, Lexington, KY). The immunoblot was developed with Renaissance brand chemiluminescence reagent (NEN Life Science Products, Boston, MA) with secondary antibody, peroxidase-labeled goat anti-rabbit IgG (Sigma). Western blotting for p38 and phospho-p38 was performed with rabbit polyclonal anti-p38 and anti-phospho-p38 antibodies (New England Biolabs), the secondary antibody, and enhanced chemiluminescence as described above. For iNOS Western blotting, the cells were exposed to IL-1β at a concentration of 10 nM (R&D Systems, Minneapolis, MN) for 24 h; for kinase assays, exposure to IL-1β was 15 min. Antibodies to c-Myc 9E10 epitope and Rho were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A nondenaturing JNK assay kit was purchased from New England Biolabs.

Preparation of nuclear extracts. Nuclear extracts were prepared as described by Dignam et al. (15) with some modifications. Cultured cells were rinsed twice with ice-cold PBS, scraped into 1 ml of PBS, and microcentrifuged at 4,500 rpm for 5 min. The pelleted cells were resuspended in 1 ml of buffer A (10 mM Tris, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 0.1 mM Na3VO4). The cells were microcentrifuged at 4,500 rpm for 5 min, resuspended in 0.5 ml of buffer A, and recentrifuged at 4,500 rpm for 5 min. The cells were resuspended and gently mixed for 10 min at 0°C in 100 ml of buffer A with 0.1% Triton X-100 to lyse the cell membranes. The nuclei were recovered by centrifugation at 7,000 rpm for 5 min. The nuclear proteins were extracted at 0°C by gently mixing the nuclei in 75 ml of buffer C (20 mM Tris, pH 7.5, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.1% Triton X-100, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 0.1 mM Na3VO4). The supernatants were collected after 25 min of microcentrifugation at 14,500 rpm for 15 min. The supernatants were diluted 1:2 in a buffer containing 1 part buffer C to 2 parts buffer D (20 mM Tris, pH 7.5, 1.5 mM MgCl2, 25 mM KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM PMSF, and 0.1 mM Na3VO4). Protein concentration was measured with the Bradford assay.

Electrophoretic mobility shift assay. Nuclear extracts (2.5 µg) were incubated with 1 µl (~50,000 counts/min) of 32P-labeled high-affinity serum-inducible element (hsSIE) oligonucleotide (~2 pM) for 30 min at room temperature in a buffer containing 2 µl (2 mg) of poly(dI-dC) (Boehringer Mannheim), 4 µl of buffer X (10 mM Tris, pH 7.5, 10% glycerol, 1 mM EDTA, 0.5 mM dithiothreitol, and 1% Nonidet P-40), 0.5 µl of buffer D, and the remainder water (final volume 20 µl). hsSIE preferentially binds STAT3 and STAT1 (46). This probe was end labeled by a fill-in reaction with the Klenow fragment of DNA polymerase I. For supershift assays, nuclear extracts were incubated on ice for 1 h with 2 µl of anti-STAT3 antibody (200 µg/0.1 ml), 2 µl of anti-STAT1 p84/p91 antibody (100 µg/ml), 1 µl of anti-STAT3 antibody, or 1 µl of anti-STAT3 antibody. DNA-protein complexes were resolved on a 4% non-denaturing polyacrylamide gel in 0.4× Tris-borate-EDTA running buffer. After electrophoresis, the gels were dried, subjected to autoradiography, and analyzed with a phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Kinase assays. Cells in 75-cm2 culture flasks were rinsed once with ice-cold PBS and then solubilized on ice for 5 min in 350 µl of lysis buffer (20 mM HEPEs, pH 7.4, 2 mM EGTA, 50 mM β-glycerophosphate, 1% Triton X-100, and 10% glycerol) plus inhibitors (1 mM dithiothreitol, 2 mM Na3VO4, 1 mM PMSF, 1 mg/ml of leupeptin, 5 mg/ml of antipain, and 1 mg/ml of aprotinin). The lysates were sonicated briefly and spun for 5 min at 10,000 rpm to remove debris. The cleared lysates were assayed for protein concentration with the Brad-
ford assay. From each condition, 250 μg of protein were incubated for 4 h on a rocking table at 4°C with agarose-conjugated antibodies. For the MAPK assay, 10 μl of goat anti-rat ERK1 and ERK2 antibodies were used for each reaction. For the p38 kinase assay, 20 μl of goat anti-human JNK1 antibody were used for each reaction. All antibodies were obtained as agarose conjugates (Santa Cruz Biotechnology). After incubation, the beads were washed three times with lysis buffer, three times with LiCl wash buffer (500 mM LiCl, 100 mM Tris-Cl, pH 7.6, and 0.1% Triton X-100 plus the inhibitors as above), and three times with assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl₂, and 0.1% Triton X-100 plus the inhibitors as above). After the final wash, the beads were left suspended in an equal volume of assay buffer to which 100 μg of substrate and a radioactive ATP mix [50 mM MgCl₂ and 0.5 mM (6,000 counts·min⁻¹·pmol⁻¹) (γ-³²P]ATP] were added. For p42/p44 MAPK and p38 MAPK, myelin basic protein was used (Sigma). For the p46/p54 kinase assay, glutathione S-transferase fusion protein was used. Reaction mixtures were incubated for 20 min at 30°C, with mixing every 2 min. The reaction was stopped with the addition of 30 μl of 5× Laemmli buffer. The samples were boiled for 2 min, spun briefly at 3,000 rpm, and separated electrophoretically on a polyacrylamide gel. The gels were dried, exposed to a phosphor screen overnight, imaged on a Storm 860 phosphorimager (Molecular Dynamics), and then exposed to Kodak Biomax MR film (Eastman Kodak, Rochester, NY).

Nonradioactive JNK assay was performed with a New England Biolabs kit, which involves an agarose-c-Jun bead to "pull down" the JNK followed by an in vitro kinase reaction, electrophoresis of the c-Jun protein, and immunoblot for phospho-c-Jun.

C3 exoenzyme treatment. Cells were treated with C3 exoenzyme with the scrape-loading technique of Malcolm et al. (28). RPASMCs were grown to confluence in 100-mm culture dishes (Corning, Corning, NY), and the monolayer was rinsed once in 5 ml of sterile PBS and once in 2 ml of scrape-loading buffer (114 mM KCl, 15 mM NaCl, and 5.5 mM MgCl₂). Each dish was then given 0.5 ml of scrape-loading buffer or 0.5 ml of scrape-loading buffer containing 5 μg/ml of Clostridium botulinum ADP-ribosyltransferase C3 (Sigma). The cells were gently scraped up into the buffer, pipetted up and down to ensure even dispersal throughout the mix, and then replated into six-well plates (Costar, Corning), plating the cells from one 100-mm dish into one well. Each well was given 3 ml of growth medium without serum; after 15 min, serum was added at the concentration found in regular growth medium. The cells were allowed to recover in growth medium overnight. Unscraped cells were serum starved overnight while the scraped cells recovered in growth medium. The next day, all cells were given IL-1β in basal medium.

ADP-ribosylation assay. Control and C3-treated cells were washed and lysed separately. The level of ADP-ribosylation was determined by incubation of the cell homogenates with additional C3 transferase (5 μg/ml) in the presence of 5 μCi of [³²P]NAD for 90 min at 30°C. The reaction was terminated by the addition of Laemmli sample buffer. After normalization for protein content, 100 μg of protein were loaded per lane, separated by SDS-PAGE, transferred to nitrocellulose membrane, and subjected to autoradiography.

Adenoviral-mediated transfer of small G proteins. To determine the proper level of adenovirus to use for our cell type, we used an adenovirus expressing LacZ (a gift from Dr. Bruce Johnson, University of Pittsburgh, Pittsburgh, PA). We found that a multiplicity of infection (MOI) of 1:1,000 was sufficient to demonstrate viral expression in 100% of the cells as determined by X-Gal staining at 48 h. Cells were grown to 80%-confluence in 10-cm petri dishes. They were infected in DMEM with a MOI of 1:1,000. A volume sufficient to cover the cells (typically 250 μl for a 25-cm² flask) containing the virus was added to the cells, and the flasks were rocked every 15 min to ensure dispersion of the virus over the surface of the dish. After 1 h, basal medium was added to increase the volume of the medium to 5 ml/T25 flask. Experiments were performed after 48 h of exposure to the virus in basal medium.

Data analysis. Pixel density determination and analysis of autoradiographs were performed with EagleSight software (Stratagene, La Jolla, CA).

RESULTS

Rho proteins but not Rac1 affect IL-1β-mediated iNOS induction in RPASMCs. From an original report by Finder et al. (16) and observations by others (19, 32), it appears that a candidate small GTPase that is geranylgeranylated at its isoprenyl moiety (e.g., Rho superfamily) is involved in the negative regulation of iNOS. Accordingly, we investigated the contribution of two members of the Rho family (Rac and Rho) to IL-1β-mediated regulation of iNOS in RPASMCs. Adenoviral-mediated infection of RPASMCs with either a dominant negative (N17) mutant of Rac1 or a constitutively active form (V12) of Rac1 did not affect either the basal levels of iNOS or the increase in immunoreactive iNOS after IL-1β (Fig. 1, top). Transgene expression was demonstrated by presence of the epitope tag c-Myc via Western blotting (Fig. 1, bottom). Infection of RPASMCs with adenoviral vectors containing cDNA for a reporter gene, β-galactosidase, of a similar MOI resulted in quantitative transgene expression (data not shown).

Fig. 1. Adenoviral transfer of Rac1 does not affect inducible nitric oxide synthase (iNOS) expression in interleukin (IL)-1β-stimulated rat pulmonary artery smooth muscle cells (RPASMCs). Monolayers of RPASMCs were infected with adenovirus expressing either the dominant negative (N17) Rac1 (AdN17Rac) or the constitutively active (V12) form (AdV12Rac). Forty-eight hours after viral infection, cells were stimulated with IL-1β (10 ng/ml). Proteins were harvested 24 h after stimulation, and immunoblot was performed for iNOS and c-Myc epitope tag (to demonstrate presence of viral infection). BM, basal medium (unstimulated control cells); IL-1, control cells treated with IL-1β. Viral treatment did not affect iNOS expression despite viral protein expression.
To assess the contribution of Rho, we used the Clostridium botulinum C3 exoenzyme that ADP-ribosylates Rho at amino acid 41 (Asn) in its effector domain and keeps the G protein in an inactive conformation. Because externally applied C3 exoenzyme does not fully penetrate cells, we used the scrape-loading technique of Malcolm et al. (28) to introduce the C3 exoenzyme into the cells. Cellular morphology seen in the cells treated with C3 exotoxin appeared to mirror that seen in cells treated with a geranylgeranyltransferase inhibitor, GGTI-298; the cytoplasm appeared to shrink to threadlike appendages attached to a rounded central nucleus (40). Nonetheless, the ability of IL-1β to stimulate iNOS was enhanced when the cells were pre-treated with C3 exotoxin. Furthermore, C3-treated cells expressed iNOS in the absence of IL-1β treatment (Fig. 2A). We performed this experiment four times and found a 29-fold (mean; range 11- to 53-fold) increase in C3-treated cells over the basal expression using computerized band densitometry software. In Fig. 2, the level of expression of iNOS in cells that had been scrape loaded and then treated with IL-1β was actually decreased relative to confluent monolayers treated with IL-1β (IL-1 sham vs. IL-1). This finding was consistent across multiple repeats of this experiment. In contrast, the level of iNOS in C3-treated, scrape-loaded cells exceeded the level seen in non-scrape-loaded cells treated with IL-1β. C3 indeed appeared to affect RhoA as shown in Fig. 2B, which demonstrates that C3 exoenzyme treatment resulted in ADP-ribosylation of the Rho proteins such that a subsequent treatment with ADP-ribosylase did not allow further attachment of ADP.

**IL-1β stimulates p44/p42 MAPK activation, but this is not required for iNOS induction.** Exposure of RPASMCs to IL-1β resulted in activation of ERK1/ERK2 MAPK as noted by an increase in immunoprecipitated p44/p42MAPK that was sensitive to the MAPK kinase 1 inhibitor PD-98059 (Fig. 3, bottom). Although IL-1β increased immunoreactive iNOS in these same cells, this increase was not sensitive to PD-98059 (Fig. 3, top), suggesting that ERK1/ERK2 kinase was not critical for IL-1β-mediated regulation of iNOS expression.

In Fig. 4, we note that stimulation of RPASMCs with IL-1β led to an increase in JNK that was not affected by the addition of either farnesytransferase (A) or geranylgeranyltransferase (B) inhibitors. Because the former inhibitor decreased and the latter inhibitor increased, respectively, IL-1β-mediated iNOS expres-
The noticeable lack of effect of these inhibitors on JNK/SAPK suggests that this pathway is not critical for the regulation of iNOS by IL-1β in RPASMCs.

**IL-1β, p38 kinase cascade, and iNOS expression in RPASMCs.** Exposure of RPASMCs to IL-1β resulted in an increase in p38 kinase as ascertained by the increase in immunoreactive phosphorylated p38 (Figs. 5 and 6). Neither GGTI-298 (Fig. 5) nor SB-203580 (Fig. 6) affected the phosphorylation status of p38 under basal or IL-1β-stimulated conditions. It is possible that GGTI-298, like SB-203580 (10), decreases the activity of p38 kinase without affecting phosphorylation status. Regardless, GGTI-298 (Fig. 5) and SB-203580 (Fig. 6) were each associated with an increase in iNOS expression after IL-1β, suggesting a possible negative role for p38 in the regulation of iNOS in RPASMCs.

**IL-1β and inhibition of geranylgeranyltransferase activate STAT3 in RPASMCs.** As shown in Fig. 7, a 15-min incubation of RPASMCs with either IL-1β or GGTI-298 resulted in the nuclear translocation of STAT1 and/or STAT3 as detected by the reduced mobility of a probe containing consensus sequences for STAT1/3 binding, i.e., hSIE (46). A combination of IL-1β and GGTI-298, however, did not lead to greater nuclear translocation than either agent alone. Excess cold hSIE oligonucleotide inhibited the formation of the nuclear complex, suggesting a degree of specificity of such translocation (data not shown). Inclusion of antisera to STAT3 but not to STAT1 in the binding reactions resulted in a further reduction in the mobility of the complex after either IL-1β or GGTI-298 (Fig. 8). The combination of IL-1β and GGTI-298, however, did not result in enhanced intensity of the supershift complex compared with either agent alone (Fig. 8). Therefore, it appears that GGTI-298, like IL-1β, can induce STAT3 homodimers, and this induction is associated with enhanced iNOS expression. The specificity of these observations was supported by the lack of effect of FTI-277 on STAT activation (data not shown).

The nuclear translocation of STAT3 was insensitive to the JAK inhibitor AG-490, suggesting that tyrosine phosphorylation of STAT3 by JAK2 after IL-1β exposure in RPASMCs was not critical. The JAK2 inhibitor AG-490 did not affect expression of iNOS under control conditions.
and IL-1β conditions and with treatment with GGTTI-298, suggesting that the STAT3 activation by either IL-1 or GGTTI-298 was not JAK2 mediated.

**DISCUSSION**

IL-1β is a critical sentinel inflammatory cytokine in the pulmonary circulation. By affecting various cell types in the pulmonary vascular wall, IL-1β is capable of altering pulmonary vasoregulation, remodeling of the vascular wall, and/or the response to other inflammatory mediators. It is apparent that pulmonary vascular smooth muscle cells are an important target for IL-1β. Although the intracellular signaling pathways responsible for IL-1β-mediated changes in pulmonary vascular smooth muscle metabolism are unclear, induction of iNOS appears to be an important consequence. The present study is an extension of the original observations by Finder et al. (16) that showed that an inhibitor of geranylgeranyltransferase (GGTI-298) superinduced iNOS after IL-1β in RPASMCs. We now show that in pulmonary vascular smooth muscle, a C3-sensitive (e.g., Rho) member of the Rho superfamily is a negative regulator of IL-1β-mediated induction of iNOS (Fig. 2). The pleiotropic IL-1β activates MAPK (ERK1/ERK2; Fig. 3); JNK/SAPK (Fig. 4); p38 kinase (Figs. 5 and 6), and JAK-STAT pathways (Figs. 7 and 8) in RPASMCs. The former two pathways, however, appear unrelated to IL-1β-mediated iNOS regulation. The p38 pathway may have inhibitory input because there is an SB-203580 (p38; Fig. 5)-dependent augmentation of IL-1β-mediated iNOS induction. As for the JAK-STAT pathway, we conclude that the association of GGTTI-298-induced STAT3 homodimer nuclear translocation provides evidence that there is upstream inhibition, likely Rho mediated, of JAK-STAT activation and that it is not JAK2 mediated. These findings suggest that the previous report by Finder et al. on the amplification of IL-1β-mediated iNOS most likely reflects downstream events, in part mediated by STAT3.

Rho as a negative regulator of IL-1β-mediated iNOS induction in RPASMCs. GGTTI-298 by itself can induce iNOS expression in RPASMCs and enhance IL-1β-mediated iNOS expression (Fig. 5), suggesting that a geranylgeranylated protein is a negative regulator of such gene regulation. This peptidomimetic inhibitor of protein isoprenylation has been shown to be extremely selective for geranylgeranyltransferase over farnesyltransferase (37, 45). We also used GGTTI-2166 where indicated. This agent has been shown to be as potent as GGTTI-298 but even more selective (41). Similarly, the farnesyltransferase inhibitor FTI-277 is also very selective for farnesyltransferase over geranylgeranyltransferase (36). The involvement of a geranylgeranylated protein in this pathway was supported by the previous observations by Finder et al. (16) that lovastatin can superinduce iNOS expression in RPASMCs and that this effect is reversed on the addition of geranylgeranoliol. Likely candidate signaling molecules that are geranylgeranylated include members of the Rho family (RhoA, RhoB, and RhoC; Rac1 and Rac2; Cdc42). We used genetic approaches to discount a possible role for Rac1 by overexpressing either dominant negative or constitutively active Rac1 and not affecting iNOS regulation (Fig. 1). We did observe, however, that C3 exoenzyme, which inactivates Rho proteins by ADP-ribosylating asparagine at amino acid residue 41 (Fig. 2B), increased basal iNOS expression and greatly enhanced the effect of IL-1β (Fig. 2A). The similarity in the morphological changes in RPASMCs exposed to C3 or GGTTI-298 (40) is additional evidence that Rho proteins play a critical role in pulmonary vascular smooth muscle cell biology. It is unclear which of the Rho proteins or other C3-sensitive members of the Rho superfamily are involved. Nonetheless, these results are quite similar to a very recent report in rat aortic smooth muscle (32) and suggest similarities rather than differences in this limited comparison of pulmonary and systemic smooth muscle cells. Rho proteins have also recently been reported (27, 42) to be important in the regulation of endothelial NOS in endothelial cells.

**IL-1β, Rho proteins, MAPK, SAPK, and p38 kinase in iNOS regulation in RPASMCs.** IL-1β is known to activate MAPKs in various cells including a number of smooth muscle cell types (3, 20, 25). Rho proteins are known to be involved in SAPK and p38 kinase pathways (17). Furthermore, ERK1/ERK2 (24, 26), JNK/SAPK (18), and p38 kinase (2, 18, 24, 26) have been noted to contribute to IL-1β-mediated increase in iNOS expression in a variety of cells including mesangial cells, cardiac myocytes, pancreatic islet cells, and chondrocytes. Although IL-1β activated the kinases ERK1/ERK2 (Fig. 3), JNK/SAPK (Fig. 4), and p38 kinase (Fig. 5), the IL-1β-mediated increase in iNOS was insensitive to PD-98059 (Fig. 3) and was actually enhanced in the presence of SB-203580 (Fig. 6), suggesting that ERK1/ERK2 was not important for this effect and that p38 kinase was an inhibitor of the IL-1β-mediated increase in iNOS in RPASMCs. Conversely, IL-1β-mediated activation of JNK/SAPK was unaffected by the presence of either a farnesyltransferase or geranylgeranyltransferase inhibitor (Fig. 4) that itself affected iNOS expression, thereby dissociating these events. Thus although Rho proteins are known to activate p38 kinase in other cell types (17), the lack of effect of GGTTI-298 on the phosphorylation status of p38 (Fig. 5) suggests that other members of the Rho GTPase family are involved. Indeed, the superinduction of iNOS by SB-203580 (Fig. 6) in IL-1β-exposed RPASMCs is relatively unique among all cytokine-induced iNOS regulation where most investigators have noted a p38-dependent increase (1, 2, 4, 5, 13, 14, 18, 24, 26, 30). This type of cell and cytokine specificity regarding regulation of iNOS is common. For example, within murine macrophages themselves, Chen and Wang (13) have noted that p38 kinase did not affect tumor necrosis factor-α or IFN-γ induction of iNOS, whereas p38 kinase was inhibitory to IFN-γ/lipopolysaccharide induction of iNOS in these same cells (11). In this latter study, there appeared to be cross talk between p38 kinases and the JNK/SAPK pathway.
pathway that is not likely to account for our results (Fig. 4).

**IL-1β, JAK-STAT, and iNOS regulation in RPASMCs.** Although IL-1β signaling is now closely associated with various MAPKs (see above) in contrast to other cytokines, there is only very modest evidence that it activates STATs (31). In RPASMCs, IL-1β is clearly associated with translocation and activation of STAT3 (Figs. 7 and 8). Because IL-1β-mediated signaling pathways involve complex cross-reactions between MAPKs and STATs, it becomes difficult to assign a direct role to STAT activation in altered gene expression. Nonetheless, it is noteworthy that AG-490, a JAK2 inhibitor, superinduced iNOS expression, suggesting that the JAK-STAT pathway may be inhibitory to IL-1β-mediated iNOS gene regulation in RPASMCs. A similar observation was made in vascular smooth muscle cells exposed to IFN-γ and lipopolysaccharide (29) in which JAK2 was inhibited pharmacologically (AG-490) and genetically (antisense). These investigators also noted an increase in iNOS expression after introducing antibodies to either STAT1 or STAT3. Indeed, the uniqueness and specificity of this inhibitory response was demonstrated by these authors in a study in which the same stimulus and signaling pathway appeared to contribute positively to iNOS induction in RAW 264.7 cells. A positive regulatory role of STATs for iNOS was also noted in IFN-γ-stimulated C6 glioma cells (34) and DLD-1 cells (23) and contributed to the IFN-γ priming of IL-1β-mediated iNOS induction in rat islet cells (21). Accordingly, as of now, vascular smooth muscle appears to have a unique JAK-STAT inhibitory role on cytokine-mediated iNOS induction. The control of iNOS is thus both species and organ specific. We feel that the findings in rodent aortic smooth muscle reported elsewhere (32) are consistent with our own findings in the pulmonary smooth muscle cell here.

In conclusion, we have noted that IL-1β results in activation of a complex network of intracellular signaling events in RPASMCs that includes activation of ERK1/ERK2, JNK/SAPK, and p38 kinases as well as a relatively novel, cell-specific activation of STAT3. A number of important inhibitory mechanisms of IL-1β-mediated iNOS expression were noted, including such roles for Rho, p38, and JAK2. The contribution of Rho proteins to early inhibitory aspects of IL-1β-mediated iNOS expression pathways may be particularly useful in pulmonary artery smooth muscle cells because Boota et al. (6) recently reported the potential for inhibitors of geranylgeranylated transferase to inhibit IL-1β-mediated superoxide anion production and enhance NO biosynthesis. Presumably, more specific molecular targets will ultimately facilitate such rational manipulation of pulmonary vascular smooth muscle cell metabolism for therapeutic purposes.

**REFERENCES**


19. Hausding M, Witteck A, Rodriguez-Pascual F, C von Eichel-Streiber, U Forstermann, and H Kleinten. Inhibition of small G proteins of the rho family by statins or Clostrid-


37. Sebti S and Hamilton AD. Inhibitors of prenyl transfersases.


AJP-Lung Cell Mol Physiol • VOL 281 • OCTOBER 2001 • www.ajplung.org